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Applicant	: Gyula Hadlaczky et al.	Art Unit	: 1638
Serial No.	: 09/724,726	Examiner	: Brent T. Page
Filed	: November 28, 2000	Cust No.	: 77202
Conf. No.	: 7776		
Title	: *ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES*		

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Michael Lindenbaum declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000, as well as the parent applications, including the earliest application U.S. Application Serial No. 08/629,822.

2) I have reviewed the Office Action, mailed May 13, 2008, in connection with the above-captioned application.

3) I received a Bachelor's degree in Biochemistry from McGill University (Montreal, Canada) in 1980. I received a Ph.D. degree in Biochemistry from McGill University (Montreal, Canada) in 1987. From 1987 to 1992, I held a post-doctoral position at the National Institute for Medical Research, London, UK. From 1992 to 1997, I held the position of Part-time Lecturer, Dept of Neurology, McGill Unversity and from 1997 to 2002, I held the positions of senior scientist and Director within the R&D group of Chromos Molecular Systems Inc. Since 2003, I have been employed by Agrisoma Bioscience where I hold the position of Vice-President of Technology Development.

4) I have more than 25 years of experience in the area of biochemistry and molecular biology, including 10 years involvement in the production of chromosomal structures using the method as taught in the instant application and parent applications, including from animal and plant cells. I have authored or co-authored over 20 publications and am listed as a co-inventor on four pending patent applications.

5) Chromos Molecular Systems, Inc., located at 220-980 W 1st St., Mailbox # 8, North Vancouver, BC, Canada V7P 3N4, an original assignee of record, is an owner of

Agrisoma Biosciences, Inc., also located at 220-980 W 1st St., Mailbox # 8, North Vancouver, BC, Canada V7P 3N4, to whom the subject matter of this application has been licensed. The licensed technology, as it relates to generation of chromosomal structures containing amplified heterochromatin, including plant sausage chromosomes and SATACs, is a cornerstone of the technologies commonly practiced by Agrisoma Biosciences, Inc. Indeed, production and use of sausage chromosomes and SATACs is the technology upon which the commercial activities of Agrisoma are focused.

6) In my capacity as Vice-President of Technology Development at Agrisoma, I oversaw a collaboration with Dr. Daina Simmonds of the Eastern Cereal and Oilseeds Research Centre of Agriculture and Agri-Food Canada, one of the federal crop research centers of the Ministry of Canada responsible for Agriculture, regarding the use of the technology described in the above-captioned application. The work was conducted by Dr. Simmonds and her research group using DNA fragments provided to her by Agrisoma, which were used in the plant transformation of soybean (*Glycine Max*). Copy number analysis was performed by Dr. Simmond's group by southern blot using standard techniques. Resulting transformed seed and embryos were sent to Agrisoma for fluorescence *in situ* hybridization (FISH) analysis. All work was done in collaboration with Agrisoma, and I conducted periodic site visits and participated in regular telephone conversations, regarding the experiments.

7) To practice the method in plants, in particular in soybean, no additional experimentation or additional guidance was required to successfully induce amplification of pericentric DNA to generate chromosomes containing amplified heterochromatin beyond what was described in the application and known to one of skill in the art at the time the application was filed. Using methods as set forth in the above-referenced application, heterologous DNA was introduced into soybean (*Glycine Max*) to produce plant SATACs, following selection and amplification of pericentric DNA. Plants were recovered and SATACs were identified. The plants were further grown under non-selective conditions to assess stability of the SATACs in selected seed.

8) The data presented in this Declaration were conducted using 26s rDNA from Arabidopsis. 26 srDNA is highly conserved in nature among plant species. A similar vector was generated containing soybean 26S rDNA, which exhibits high homology to the Arabidopsis 26S rDNA. The pericentric amplification events, including generation of sausage chromosomes and SATACs, achieved by use of either targeting sequence were

virtually identical (data not shown). These results demonstrate that the rDNA sequence employed in the method is not plant specific.

A description of the above-referenced methods and the resulting amplification leading to the production of plant sausage chromosomes and SATACs in soybean is described in the following sections.

I. MATERIALS AND METHODS

A. Heterologous DNA (core vector)

The heterologous DNA included a construct containing a plant promoter linked to a selectable marker gene conferring resistance to the antibiotic hygromycin. The hygromycin resistance sequence is found in the pFF plasmid family (see *e.g.*, Timmermans *et al.* (1990) *J Biotechnol.* 14:333-44). The vector also included an *att B* recombination site between the 35S promoter and selectable marker (see figure 1). To generate the 35S-attB HygR cassette, a 1077 base pair SmaI fragment containing the coding sequence for the bacterial Hygromycin resistance (HygR) gene was excised from the plasmid pHyg (provided by Dr. Daina Simmonds; see also Sugden *et al.* (1985) *Mol. Cell Biol.* 5:410-413) and was gel purified. Subsequently, the intermediate plasmid pABI1012 (see Exhibit A; which includes a construct containing a CaMv 35S promoter fused to a phosphinothricin acetyl transferase gene (*bar)* as a selectable marker (White *et al.* (1989) *Nucleic Acids Res*., 18:1062), with an *att B* recombination site between the 35S promoter and *bar* selection gene contained in a pBluescript backbone (Stratagene, La Jolla, CA)), was digested with Xho1, deleting the BAR gene sequence. The digested plasmid was treated with Klenow to fill in the XhoI ends and the resulting 3914 base pair vector fragment ligated to the Smal HygR gene fragment, yielding the plasmid pABI034 containing the HygR gene inserted downstream of and in the sense orientation with respect to the 35S promoter and attB site. The vector map of pAB1034 is set forth in Figure 1. Before use, the plasmid was digested with NotI and a 2.1 kb fragment (containing the 35S promoter-attB-HygR cassette for use in transformations) was gel purified away from other plasmid sequences.

B. Targeting DNA to the pericentric region

A targeting DNA molecule was constructed to target the integration of the selectable marker to the pericentric region, in particular the pericentric rDNA, of an acrocentric chromosome. The coding region of the 26S rDNA was chosen as a targeting sequence because it is highly conserved among species and it encodes a structural RNA molecule highly conserved among eukaryotic organisms, (see, *e.g.*, Genbank Accession no. X52320,

which was deposited in the early 1990s; see also Pruit and Meyerowitz (1991) J Mol Biol., 187:169-83; Genbank Accession no. X15550; Gruender et al. (1991) J Mol Biol. 221:1209-1222). The plasmid pJHD2-19a, containing a portion of the Arabidopsis 26SrDNA gene, as described by Doelling et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:7528-7532, was digested with XhoI and a 1.5 kb fragment containing the rDNA segment was gel purified away from plasmid sequences. The vector map for pJHD2-19a is set forth in Figure 2.

C. Introduction of DNA into Glycine Max

For transformation of Glycine Max (soybean), the DNA fragment encoding the selectable marker was mixed with a 10-fold excess of targeting DNA. The DNA mixture was subsequently used for biolistic transformation of embryogenic soybean cultures cultures {see *e.g.*, Finer, J. and Nagasawa, A. (1988) Development of an embryogenic suspension culture of soybean [Glycine max (L.) Merrill]. Plant Cell. Tiss. Org. Cult. 15, 125-136; Finer, J. J. and Mcmullen, M. D. (1991) Transformation of soybean via particle Bombardment of embryogenic suspention culture tissue. In Vitro Cell. Dev. Biol. 27, 175-182.}. After selection in hygromycin containing medium, resistant soybean calli were obtained and genomic DNA isolated for standard copy number analysis via Southern blot for the presence of the introduced DNA containing the selectable marker.

The plants were grown under non-selective conditions by culturing transgenic embryogenic clusters in the absence of selective agent under standard conditions established for the establishment of somatic embryos {see *e.g.* Finer and McMullen (1991). Transformation of soybean by particle bombardment of embryogenic suspension culture tissue. In Vitro Cell Dev Biol-Plant 27:175-182.}. Mature embryos were desiccated in darkness at 20°C for 5-7 days then germinated. Within a few days the roots emerged followed by shoots. Germinated embryos were planted in artificial soil and placed in a controlled growth cabinet at 26/24°C (day/night), 18h photoperiod. At the 2 -3 trifoliate stage (2-3 weeks) the plantlets were transplanted to 12.5-cm fiber pots and grown at 18-h photoperiod. The photoperiod was reduced to 13h for flower induction (approx. 2 weeks after potting).

Fluorescent in situ hybridization (FISH) was performed on root tips of embryos regenerated from the primary transgenic calli (carried out in the absence of hygromycin selection) or T1 seeds obtained from self crosses of fully regenerated transgenic plants which were germinated in the absence of hygromycin selection.. Metaphase chromosome spreads were prepared from mitotically blocked root-tips and hybridized with a combination of labeled probes directed to the core vector sequence (green signal) as well as the endogenous

45S nuclear rDNA gene locus (red labeled) for detection of the pericentric region. The rDNA probe used was directed against an adjacent region of the loci (18S) to that used for the targeting sequence (26S). As a control for probe signal, FISH analysis also was performed on wild-type somatic embryos or seeds that were not transformed

II. RESULTS

A. Copy Number

Following introduction of heterologous DNA into *Glyine Max*, calli were selected for hygromycin-resistant events. Table 1 depicts a summary of the number of hygromycin-resistant events obtained following transformation of soybean with the DNA fragments. Genomic DNA isolated from the resistant calli were used for southern blot to assess whether the DNA containing the selectable marker that was introduced was amplified. As can be seen in the Table below, a significant number of the total events contained multi-copy insertions of the core vector (between 30% and 48% of events per transformation experiment).

Transformation series	Number of events obtained	Copy number	
7B8	32	Greater than 10 copies:	13 events
		Between 4 and 10 copies:	13 events
		Less than 4 copies:	6 events

B. Florescence in situ hybridization (FISH)

To determine whether the DNA containing the heterologous sequences (including a bacterial hygromycin resistance gene) was co-integrated into an endogenous rDNA locus, and was capable of eliciting amplification of pericentric heterochromatin and subsequent SATAC formation, fluorescence *in-situ* hybridization (FISH) was performed. Metaphase chromosome spreads from T1 seed-derived root tips of event 7B8 6B17 self crosses (containing 33-35 copies of core vector sequence by southern blot analysis) were hybridized with a mixture of probes directed against core vector pABI034 insert (Texas red labeled) and 18SrDNA (Alexafluor green labeled). The results showed that wildtype soybean nuclei contain 2 rDNA loci (one per haploid genome) that hybridize with the complementary rDNA probe (red) (data not shown). This is consistent with the known distribution of rDNA loci of soybean which have been reported to contain a single 45S DNA locus (2 per diploid genome; Skorupska *et al.* (1989) Detection of ribosomal RNA genes in soybean, *Glycine max* (L) Merr., by *in-situ* hybridization. *Genome*, 32:1091-1095)) in the short arm region of

acrocentric chromosome 13 (Griffor *et al.* (1991) Fluorescent in-situ hybridization to soybean metaphase chromosomes, *Plant Mol. Biol.*, 17:101-109). Note that there is no apparent hybridization to core vector probe (green), consistent with the expectation that these heterologous sequences have no highly related counterparts in the wildtype soybean genome (data not shown).

A SATAC consisting of highly co-ampliifed pericentric rDNA and core vector (heterologous DNA) sequences was observed in multiple spreads (see Figure 3a, d, g for examples of such spreads) consistent with the described structure of a SATAC. No other cellular chromosomes were observed to contain core vector sequences and, apart from the SATAC, only two host acrocentric chromosomes were found to contain abundant rDNA in the pericentric short arm regions consistent with the known distribution of rDNA loci of soybean. Analysis of hybridized spreads at higher magnification (figures 3B and C, 3E and F, 3 H and I; B, E and H show the Texas red and Alexafluor Green signals of the SATAC while images C, F and I show the same SATAC stained with DAPI) show that it is clear that the SATAC is, in fact, separate and autonomous of other cellular chromosomes.

III. CONCLUSION

The data provided in this Declaration were generated by practice of the method in soybean plants exactly as described in the application, including introduction of a DNA fragment containing a selectable marker into soybean and growth of the calli under selective conditions. SATACs were identified in seeds obtained from regenerated plant cells grown under non-selective conditions. The SATACs were identified by co-localization of amplified pericentric DNA and heterologous DNA. The SATAC also was identified as being a separate and autonomous chromosome. Further, the results evidence the stability of the SATAC, even under non-selective conditions. The 7B8 6B17 T0 plants were regenerated and T1 seeds germinated in the absence of hygromycin selection, showing that the plant SATAC has a fully functional centromere that permits it to undergo mitosis in somatic cells as well as meiotic replication enabling it to enter the germ line and be sexually transmitted to the T1 progeny. Thus, by following the teachings in the above-captioned application one can readily generate chromosomal structures, including SATACs, following introduction of DNA into soybean, selection and amplification of pericentric DNA.

I further declare that all statement made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

November 26, 2008
Date

Michael Lindenbaum
Michael Lindenbaum

Figure 1: Core vector containing selectable marker

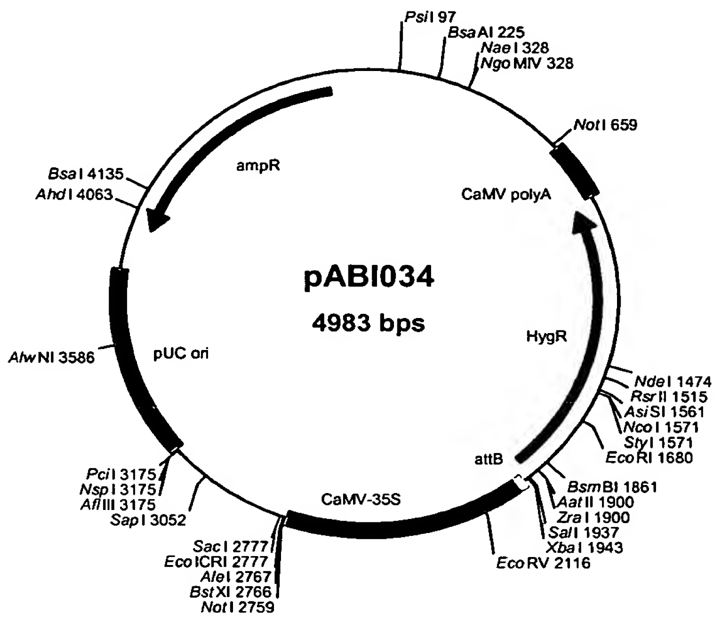

Figure 2: Vector containing 26S rDNA sequences cloned from Arabidopsis (pJHD2-19a)

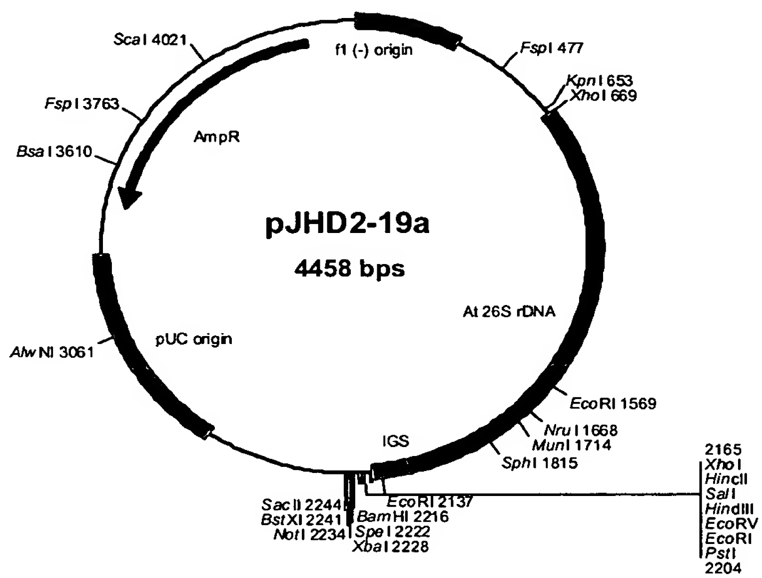

Figure 3: FISH analysis of T1 metaphase nuclei of transgenic event 7B8-6B17

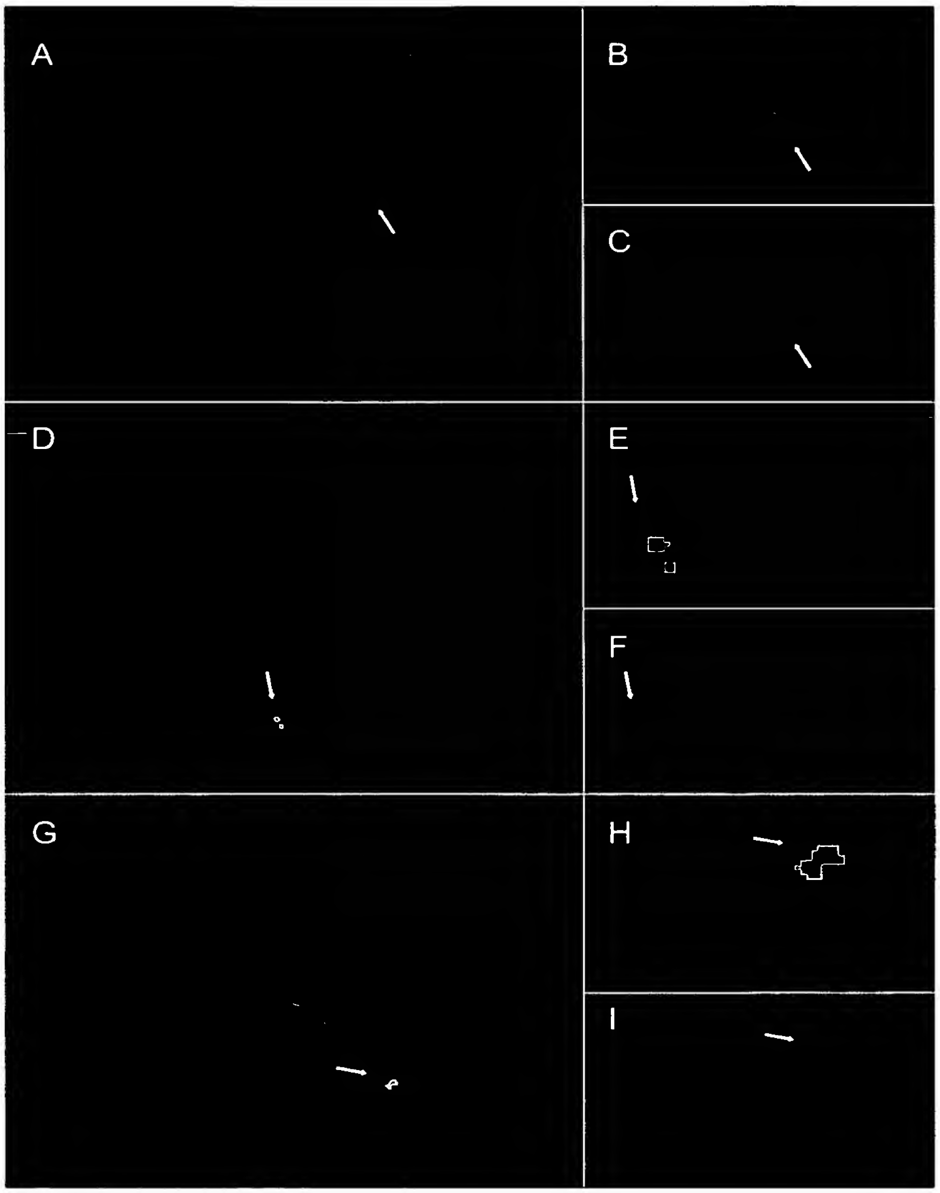

Exhibit A: Annotated Nucleotide Sequence of pABI 012 Selectable Marker Vector

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   1  CGACACTCTC GTCTACTCCA AGAATATCAA AGATACAGTC TCAGAAGACC AAAGGGCTAT TGAGACTTTT CAACAAAGGG TAATATCGGG AAACCTCCTC GGATTCCATT
      >>......................................................CaMV-35S.......................................................>

 111  GCCCAGCTAT CTGTCACTTC ATCAAAAGGA CAGTAGAAAA GGAAGGTGGC ACCTACAAAT GCCATCATTG CGATAAAGGA AAGGCTATCG TTCAAGATGC CTCTGCCGAC
      >.......................................................CaMV-35S.......................................................>

 221  AGTGGTCCCA AAGATGGACC CCCACCCACG AGGAGCATCG TGGAAAAAGA AGACGTTCCA ACCACGTCTT CAAAGCAAGT GGATTGATGT GATAACATGG TGGAGCACGA
      >.......................................................CaMV-35S.......................................................>

 331  CACTCTCGTC TACTCCAAGA ATATCAAAGA TACAGTCTCA GAAGACCAAA GGGCTATTGA GACTTTTCAA CAAAGGGTAA TATCGGGAAA CCTCCTCGGA TTCCATTGCC
      >.......................................................CaMV-35S.......................................................>

 441  CAGCTATCTG TCACTTCATC AAAAGGACAG TAGAAAAGGA AGGTGGCACC TACAAATGCC ATCATTGCGA TAAAGGAAAG GCTATCGTTC AAGATGCCTC TGCCGACAGT
      >.......................................................CaMV-35S.......................................................>

 551  GGTCCCAAAG ATGGACCCCC ACCCACGAGG AGCATCGTGG AAAAAGAAGA CGTTCCAACC ACGTCTTCAA AGCAAGTGGA TTGATGTGAT ATCTCCACTG ACGTAAGGGA
      >.......................................................CaMV-35S.......................................................>

 661  TGACGCACAA TCCCACTATC CTTCGCAAGA CCTTCCTCTA TATAAGGAAG TTCATTTCAT TTGGAGAGGA CACGCTGAAA TCACCAGTCT CTCTCTACAA ATCTATCTCT
      >.......................................................CaMV-35S......................................................>>
                                                                                                                       Att B <

 771  GTCGAGTGAA GCCTGCTTTT TTATACTAAC TTGAGCGAAC TCGAGTCTAC CATGAGCCCA GAACGACGCC CGGCCGACAT CCGCCGTGCC ACCGAGGCGG ACATGCCGGC
      <.....................Att B....................<<
                                                              >>............................Bar..............................>

 881  GGTCTGCACC ATCGTCAACC ACTACATCGA GACAAGCACG GTCAACTTCC GTACCGAGCC GCAGGAACCG CAGGAGTGGA CGGACGACCT CGTCCGTCTG CGGGAGCGCT
      >.........................................................Bar..........................................................>

 991  ATCCCTGGCT CGTCGCCGAG GTGGACGGCG AGGTCGCCGG CATCGCCTAC GCGGGCCCCT GGAAGGCACG CAACGCCTAC GACTGGACGG CCGAGTCGAC CGTGTACGTC
      >.........................................................Bar..........................................................>

1101  TCCCCCCGCC ACCAGCGGAC GGGACTGGGC TCCACGCTCT ACACCCACCT GCTGAAGTCC CTGGAGGCAC AGGGCTTCAA GAGCGTGGTC GCTGTCATCG GGCTGCCCAA
      >.........................................................Bar..........................................................>

1211  CGACCCGAGC GTGCGCATGC ACGAGGCGCT CGGATATGCC CCCCGCGGCA TGCTGCGGGC GGCCGGCTTC AAGCACGGGA ACTGGCATGA CGTGGGTTTC TGGCAGCTGG
      >.........................................................Bar..........................................................>

1321  ACTTCAGCCT GCCGGTACCG CCCCGTCCGG TCCTGCCCGT CACCGAGATT TGACTCGAGT TTCTCCATAA TAATGTGTGA GTAGTTCCCA GATAAGGGAA TTAGGGTTCC
      >..........................Bar..........................>>
                                                                     >>.....................CaMV polyA.......................>

1431  TATAGGGTTT CGCTCATGTG TTGAGCATAT AAGAAACCCT TAGTATGTAT TTGTATTTGT AAAATACTTC TATCAATAAA ATTTCTAATT CCTAAAACCA AAATCCAGTA
      >......................................................CaMV polyA......................................................>

1541  CTAAAATCCA GATCCCCCGA ATTAATTCGG CGTTAATTCA G
      >................CaMV polyA................>>
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